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13. ABSTRACT *(Maximum 200 Words)*

This study was designed to identify the RA target genes in the normal human mammary epithelial cells (HMEC) and breast cancer cells. We found that the IL-1β gene was an early responsive gene and its expression was up-regulated as early as two hours after RA treatment. Results from the treatment of HMEC with cycloheximide and actinomycin D indicated that the expression of the IL-1β gene was regulated by RA at the transcriptional level and that the regulation does not require the concurrent protein synthesis. Either RA or IL-1β could significantly inhibit the proliferation of HMEC. However, the addition of soluble IL-1 receptor antagonist (sIL-1ra) to the cell culture medium did not block RA-induced HMEC growth inhibition, whereas sIL-1ra blocked the growth inhibition of HMEC by IL-1β. The basal IL-1β expression was lost in the human breast carcinoma MCF-7, MDA-MB-231 and MDA-MB-468 cell lines. The strong inhibitory effects of RA and IL-1β on the growth of the estrogen receptor (ER) positive MCF-7 cell line were shown, but only a small effect on the ER negative MDA-MB-231 cells was found. Our study supports the hypothesis that the co-administration of IL-1β may enhance the therapeutic effect of RA on certain breast cancers.

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Table of Contents

Cover.....I

SF 298.....II

Table of Contents.....III

Introduction.....1

Body.....1

Key Research Accomplishments.....23

Reportable Outcomes.....23

Conclusions.....23

References.....25

INTRODUCTION

Retinoic acid (RA) and some of its synthetic derivatives can act as chemopreventive agents for certain malignant diseases, including breast cancers. At a molecular level, RA acts via binding and activating its nuclear receptors, which are transcription factors that directly regulate the transcription of certain "target" genes. To understand the chemopreventive mechanisms of RA in the process of breast carcinogenesis, the downstream target genes of RA nuclear receptors must be identified. The identification and characterization of RA target genes in cultured *normal* human breast epithelial cells are the major goals in the current proposal. The comprehensive characterization of RA target genes will increase our understanding of the molecular mechanisms which underly the RA associated growth arrest and differentiation of human mammary epithelial cells (HMEC). These studies will also provide an important foundation for designing more specific and more effective agents for the prevention and therapy of breast carcinoma, and potentially for the other types of carcinomas as well. The major goals of this project include: 1. To identify the genes regulated by RA in normal breast epithelial cells; 2. To determine how RA affects the expression of its target genes; 3. To characterize the functions of the isolated RA target genes on the growth and differentiation of HMEC; 4. To compare the effects of RA on the expression of RA target genes and on the growth of human mammary epithelial cells and breast cancer cells; 5. To monitor the gene expression profile in HMEC after RA treatment by using oligo-microarray analysis. In the current report, we include the result of the characterization on one of the RA target genes, the interleukin-1β (IL-1β) gene. Our study supports the hypothesis that the co-administration of IL-1β may enhance the therapeutic effect of RA on certain breast cancers.

BODY

Retinoic acid (RA) regulates gene expression via binding to its nuclear receptors, the RARs, which are transcription factors that directly modulate the transcription of primary target genes (Langston and Gudas 1992; Allenby, Bocquel et al. 1993; Boylan, Lufkin et al. 1995; Faria, Mendelsohn et al. 1999). The RARs constitutively bind to the regulatory DNA elements of their target genes, but the subsequent effect on gene transcription is ligand binding-dependent (Chambon 1994). The ligand-dependent recruitment of chromatin-remodeling proteins plays an important role in the functional modulation of RA receptors (Xu, Glass et al. 1999). In the absence of ligands, RA receptors associate with a histone deacetylase (HDAC)-containing complex on the target promoters and suppress gene transcription (Nagy, Kao et al. 1997). Upon ligand binding, the repressive complex is disrupted and a histone acetyltransferase (HAT) is recruited by several co-activators, leading to transcriptional activation of the target genes (Korzus, Torchia et al. 1998; Glass and Rosenfeld 2000). Abnormal metabolism of retinoids and reduced expression of one of the RA receptors, RARβ, have been observed in malignant cells, as well as in the transition process from pre-malignant lesions to aggressive carcinomas (Hu, Crowe et al. 1991; Jing, Zhang et al. 1996; Lotan 1996; Guo, Ruiz et al. 2000; Xu, Wong et al. 2001). Since RA and its synthetic derivatives can induce the expression of one of the RARs, RARβ, and can also activate the

receptors, retinoids have been regarded as major therapeutic and chemopreventive agents for many types of cancers, including human breast cancers (Lotan 1996; Hong and Sporn 1997; Miller 1998; Alberts D.S. and al. 1999).

RA and its synthetic derivatives (retinoids) can regulate the proliferation of many types of cells (Love and Gudas 1994; Niles 2000). It has been shown that RA can influence several cell cycle regulatory proteins, but the mechanism by which growth arrest is achieved by RA may be different in different cell types. For example, in the human neuroblastoma SMS- KCNR cell line, treatment with RA increases the Cdk inhibitor p27kip1 protein expression and enhances the binding of the G1 cyclin/Cdk to p27kip1, thereby inducing cell growth arrest (Matsuo 1998). In human bronchial epithelial cells, RA enhances the degradation of the cyclin D1 protein, followed by cell growth arrest in the G1 phase of the cell cycle (Langenfeld, Kiyokawa et al. 1997). Cyclin D3 transcripts are reduced in F9 cells following RA treatment (Faria, LaRosa et al. 1998; Li, Glozak et al. 1999), and ectopic overexpression of cyclin D3 blocks the RA-induced growth arrest of F9 cells, suggesting that the reduction in cyclin D3 expression is a necessary step in the RA-induced growth inhibition of F9 cells (Faria, LaRosa et al. 1998).

In both cultured normal human mammary epithelial cells (HMEC) and estrogen receptor (ER) positive MCF-7 breast carcinoma cells, as in some other cell types, cyclin D1 has been shown to play an important role in the RA-induced growth arrest of both. The cyclin D1 protein level in HMEC decreases during the initiation of growth arrest at 24 h after RA treatment, and HMECs that express exogenous cyclin D1 are resistant to RA-induced differentiation (Seewaldt, Kim et al. 1999). In MCF-7 cells cyclin-dependent kinase 2 (cdk2) and cyclin D1 mRNA levels are reduced by 8 hours and 48 hours, respectively, following RA treatment (Teixeira and Pratt 1997), but the details of the signal transduction pathway that leads to RA-induced growth arrest of mammary epithelial cells remain unclear.

The cytokine IL-1β is secreted by a wide variety of cells. IL-1β regulates cellular activities via binding to IL-1 receptors (Watkins, Hansen et al. 1999). Two types of receptor, the IL-1 receptor type I and IL-1 receptor type II, are expressed on the plasma membrane, but only ligand-bound IL-1 type I receptor can trigger the intracellular signal transduction pathway (Dinarello 1996). Some cells express the endogenous IL-1 receptor antagonist (IL-1ra), which can inhibit the effects of IL-β by specifically blocking the IL-1 receptor (Arend, Malyak et al. 1998; Dinarello 2000). Ligand-bound IL-1 receptor activates mitogen-activated protein (MAP) kinases and IκB kinase, and subsequently transcription factors such as AP-1 and nuclear factor-κB (NF-κB) are activated, followed by cell growth arrest and apoptosis (Ichijo 1999).

The regulation of the expression of the gene IL-1β is a complicated process comprised of multiple regulatory events occurring at different levels (Watkins, Hansen et al. 1999). Many DNA regulatory elements, including the LPS response enhancer, the cAMP response element, the AP-1/NFκB site, and the negative glucocorticoid response element, have been found in the upstream regulatory region of IL-

1β gene. Although a functional retinoic acid response element (RARE) in the promoter of the IL-1β gene has not yet been delineated, it was reported that transcription of the IL-1β gene in certain cells, such as HL-60, NB-4, and healthy peripheral blood mononuclear cells (PBMC), is regulated by RA (Jarrous and Kaempfer 1994; Matikainen, Tapiovaara et al. 1994). In alveoloar macrophages it was shown that RA enhanced IL-1β and inhibited IL1ra production (Hashimoto, 1998 #56).

The present study was designed to identify genes that were positively regulated by RA in cultured normal human mammary epithelial cells. We found that the IL-1β gene is transcriptionally regulated by RA in HMEC. Our data also indicate that the IL-1β gene is a direct, downstream target gene of RA receptors in human mammary epithelial cells.

MATERIALS AND METHODS

Materials

All-*trans* retinoic acid, cycloheximide and actinomycin D were from Sigma (St Louis, MO). Recombinant interleukin-1β was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The pGEX-5X-1 plasmid that carries soluble IL-1 receptor antagonist (sIL-1ra) cDNA was a generous gift from Dr. Arend (Eisenberg, Evans et al. 1990) at University of Colorado Health Science Center.

Cells and culture conditions

Normal human mammary epithelial cells (HMEC) and normal human prostate epithelial cells (PrEC) were purchased from Clonetics Corp. (Walkersville, MD). The normal human epithelial cells from floor of the mouth (OKF4) and oral carcinoma cell SCC-25 cell line were obtained from Dr. J. Rheinwald. The breast carcinoma cell lines MCF-7, MDA-MB-231 and MDA-MB-468 were purchased from ATCC (Arlington, VA). The human prostate carcinoma Ln30 and PC-3 lines were obtained from Dr. D. Nanus at Weill Medical College of Cornell University. For maintenance of cell strains, HMEC and PrEC were cultured in mammary epithelial growth medium (MEGM) and prostate epithelial growth medium (PrEGM), respectively (Clonetics). The OKF4 cells were grown in keratinocyte SFM medium (Life Technologies, Grand Island, NY). MCF-7, MDA-MB-231 and MDA-MB-468 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5 μg/ml insulin. OKF4 and SCC-25 were cultured in keratinocyte serum-free medium (Life Technologies). Ln30 and PC-3 cells were cultured in RPMI (Life Technologies) plus 10% FCS.

Synthesis of the HMEC cDNA library and subrractive screening

Poly A+ messenger RNA was isolated from HMEC using a Poly(A) mRNA isolation kit from Stratagene (Cedar Creek, TX). A cDNA library was synthesized using a ZAP Express cDNA library synthesis kit (Stratagene) according to the manufacturer's instructions. Approximately 1.0×10^6 primary plaque-forming units (pfu) were amplified and an aliquot containing 0.2×10^6 pfu of the amplified library was plated for the differential screening by the subtracted cDNA pools (see below).

The cDNA subtraction was performed using the PCR-select cDNA subtraction kit (Clontech). Two cDNA pools, which were enriched with RA up-regulated genes and down-regulated genes, respectively, were produced by subtractive hybridization between the cDNA samples made from 6 hour-RA treated vs. untreated HMEC cells. cDNA (60 ng) from each cDNA pool was labeled with [^{32}P]-dCTP using a random primer labeling kit (Roche, Indianapolis, IN) and used for differential screening of the HMEC cDNA library. Differentially expressed clones were picked from primary screening and then further purified by secondary and tertiary screening. All purified cDNA clones were subjected to DNA sequencing. 12 genes were identified as being RA regulated in the initial subtractive hybridization screening.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was extracted from each cell line using RNA Stat-60, according to the manufacturer's instructions (Tel-Test, Friendswood, TX). RNA was fractionated by size on 1% agarose and then transferred to nylon filters. The transferred RNA was immobilized using a UV Stratalinker 1800. All cDNA probes were labeled with [^{32}P]dCTP using a random primer labeling kit (Roche). The probes were IL-1β, IL-1 receptor type I (Incyte Genomics, St. Louis, MO), IL-1 receptor type II (Incyte Genomics), and GAPDH. Blots were prehybridized in 50% formamide, 5X SSC, 5X Denhardt's, 5 mM EDTA, 10% dextran, 0.5% SDS, and 100 μg/ml salmon sperm DNA at 42°C for 6 hours. Hybridization was carried out at 42°C for 16 hours using a radiolabeled probe (1 x 10^6 cpm/ml). The blots were washed twice in 2X SSC, 0.5% SDS for 20 min at room temperature and twice in 0.2X SSC, 0.1% SDS for 20 min at 60°C. Autoradiography was performed overnight unless otherwise indicated. Quantitative analyses of Northern blots were performed using NIH Image 1.62.

Cell Proliferation Assays

Proliferation assays were performed as described previously (Hoffman, Engelstein et al. 1996). Cells were plated in 24 well tissue culture plates in appropriate medium. The medium was replaced every other day. The cells were trypsinized and counted every day using a Coulter Counter through day 6 (except MDA-MB-231 cells, which repeatedly showed marked cell death on day 6 because of high density; data through day 5 were presented in this study). Triplicate wells were counted for each time point. The data were analyzed with a Prism program.

Expression and Purification of GST-sIL-1ra Fusion Protein

The BL-21 *E. coli* strain was transformed with pGEX-5X-1/sIL-1ra plasmid. To express the protein, the transformed bacteria were incubated overnight in 2 ml Luria Broth (LB). The fresh overnight culture was diluted 1:20 into 20 ml and incubated at 37°C until OD_{600nm} reached 0.6. Expression was then induced with 1mM IPTG for 5 hours. After induction, the *E. coli* were collected by centrifugation at 1,000 x g for 10 min at 4°C. The *E. coli* pellet was resuspended with 1 ml of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and subjected to sonication (Ultrasonic Model W-370 sonicator, 3.0 setting). After centrifugation at 14,000 x g for 5 min at 4°C, the supernatant was

incubated with shaking with 20 μl (bed volume) of glutathione Sepharose beads (Pharmacia) for 30 min at 4°C. The glutathione Sepharose beads were washed with PBS 5 times. The GST-sIL-1ra protein was eluted in 50 μl of elution buffer (10 mM reduced glutathione, 50 mM Tris.HCl, pH 8.0). The concentration of the purified fusion protein was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The freshly expressed GST-sIL-1ra protein was added in the cell culture medium at 200 ng/ml for the experiment.

RESULTS

Expression of IL-1β mRNA in HMEC after RA Addition. Among RA-regulated DNAs isolated from the HMEC cDNA library, one had a DNA sequence identical to the human IL-1β gene. To confirm that RA increased the level of IL-1β mRNA in HMEC, total RNA was extracted from RA-treated versus untreated HMEC, and Northern blot analysis was performed using the human IL-1β cDNA fragment (1.4 kb) as probe. The results of these Northern blot analyses are shown in Figure 1A, and this data suggested that the IL-1β gene could be an early RA responsive gene. A marked increase in IL-1β mRNA (1.5 kb) was found at 2 hours after RA treatment (the IL-1β mRNA levels were not examined at earlier times). Quantitative analyses indicated that IL-1β mRNA levels were increased 2.3-fold at 2 hours and 3.0-fold at 4-hour, respectively, after RA treatment (Figure 1C).

To determine if the increased IL-1β mRNA levels after RA addition resulted from the enhanced gene transcription or reduced degradation of existing IL-1β transcripts, we treated the HMEC plus or minus RA, and with or without the transcription inhibitor actinomycin D, and subsequently performed Northern blot analyses. In Figure 1B, we show that when gene transcription is blocked by actinomycin D, the RA-associated increase in IL-1β mRNA level is completely abolished. Thus, the RA-associated increase in IL-1β mRNA results from increased transcription of the IL-1β gene. The protein synthesis inhibitor cycloheximide did not block the RA induced expression of IL-1β gene, indicating that concurrent protein synthesis is not required for the transcriptional activation of the IL-1β gene by RA (Figure 1A, C).

The Growth of HMEC after Culture in the Presence of RA, IL-1β, and/or GST-sIL-1ra, an IL-1 Receptor Antagonist. IL-1β regulates the phenotype of its target cells through binding to the IL-1 receptors. HMECs express both IL-1 receptor type I (4.9 kb) and IL-1 receptor type II (1.3 kb) (Figure 2A). Therefore, the IL-1β produced by HMEC could potentially function in an autocrine manner. To determine whether the HMECs respond to IL-1β, we treated HMECs with recombinant human IL-1β protein and RA, and the growth curves in the presence of RA (1 μM) or IL-1β (100 pg/ml), or both are presented in Figure 2B. The results show that growth of HMECs was significantly inhibited by either RA or IL-1β treatment. The growth inhibition was even more marked when HMECs were treated with RA plus IL-1β. By day 6, the growth of HMECs was inhibited by 62% in the presence of IL-1β, 80% in the presence of RA, and 89% in the presence of RA plus IL-1β, as compared to the control HMECs.

Since the expression of the IL-1β gene was up-regulated by RA, we next examined whether RA- induced growth inhibition of HMEC was affected when the IL-1 receptors were blocked. We expressed the recombinant soluble IL-1 receptor antagonist (sIL-1ra) as a GST fusion protein. The purified GST-sIL-1ra fusion protein was then added to the cell culture medium in order to block the IL-1 receptors by competing with IL-1β for binding to the IL-1 receptors. The cells were counted for the 6 days following the treatments (Figure 2C). The sIL-1ra alone did not significantly affect the growth rate of HMEC as compared to the control group. The effect of exogenous IL-1β (100 pg/ml) on the growth of HMEC was largely abolished by adding GST- sIL-1ra (200 ng/ml) in the cell culture medium, but the GST-sIL-1ra did not block the inhibitory effect of RA (1μM) on the growth of HMEC. Thus, we conclude that while IL-1β can inhibit the growth of HMEC cells, RA does not function via this IL-1β pathway as the sIL-1ra does not block the RA associated growth inhibition.

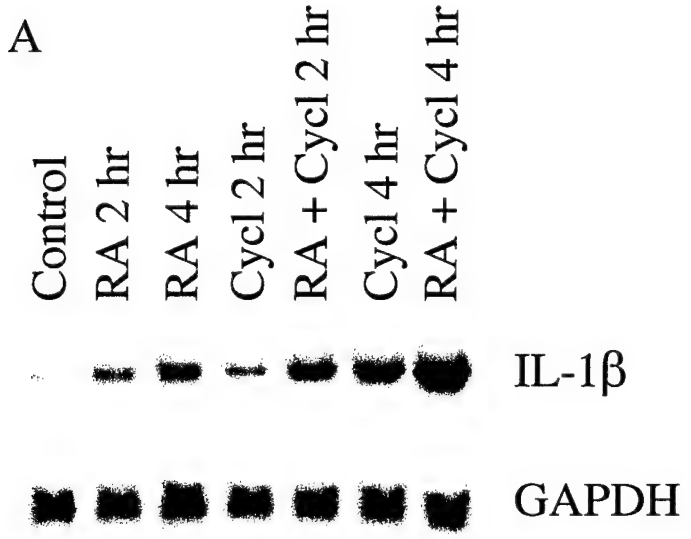
The Expression of the IL-1β Gene in Human Breast Cancer Cell Lines. To examine whether RA induced the expression of the IL-1β gene in human breast carcinoma cells, total RNA was extracted from three breast carcinoma cell lines, one which was estrogen receptor (ER) positive (MCF-7) and two which were ER negative (MDA-MB-231 and MDA-MB-468 cells). The results from Northern analyses indicated that the expression of the IL-1β gene was largely abolished in all three breast carcinoma cell lines (Figure 3A). To confirm that the distinct expression in the normal cells and the carcinoma lines was not a result of the different medium used to culture these different types of cells, we repeated this experiment culturing both the normal and the carcinoma cells in a consensus medium (1/2 MEGM + 1/2 DMEM + 5% FCS), and same results were obtained (data not shown). Upon very long exposure (14 days) of the Northern blots, an increase in the expression of the IL-1β gene in the MDA-MB-231 carcinoma line could be detected, but no expression of the IL-1β gene was detected on the same blot in the MCF-7 and the MDA-MB-468 lines (Figure 3B).

The Growth of MCF-7 and MDA-MB-231 lines after Culture in the Presence of RA and/or IL-1β. Both the MCF-7 and MDA-MB-231 cell lines expressed IL-1 receptor type I (Figure 2A). To determine whether the breast carcinoma cell lines responded to IL-1β, growth curves of ER positive MCF-7 and ER negative MDA-MB-231 cells lines in the presence of recombinant IL-1β (100 pg/ml) or RA (1 μM), or both were performed. The results (Figure 4) indicated that the growth of MCF-7 cells was inhibited by either RA or IL-1β treatment alone. Moreover, the inhibitory effects on MCF-7 cell growth were enhanced by the combined treatment of RA plus IL-1β (Figure 4A). As compared to the MCF-7 cell line, the inhibitory effects of treatment with RA, IL-1β, or RA plus IL-1β on the growth of MDA-MB-231 cells were small (Figure 4B). A comparison of growth inhibition of HMEC, MCF-7, and MDA-MB-231 cells by RA, IL-1β or both signaling molecules together during the entire course of treatment is shown in Figure 5A-C.

The Expression of the IL-1β Gene in Other Types of Cultured Human Epithelial Cells. To determine whether the RA-induced expression of IL-1β occurred in other epithelial cells, we examined

the expression of the IL-1β gene in two normal epithelial cell strains from other tissues, PrEC cells from prostate epithelium and OKF4 cells from the oral cavity. The induction of IL-1β gene expression by RA also occurred in normal prostate epithelial cells (PrEC) and in the epithelial cells of the oral cavity strain, OKF4.

To determine whether, as we had observed for breast carcinomas (Figure 3), the expression of the IL-1β gene was lost in other types of carcinomas, we examined IL-1β gene expression in the prostate carcinoma lines PC-3 and LN-30 and the oral cavity derived SCC-25 carcinoma cell line by Northern blot analysis. The expression of the IL-1β gene and the IL-1β increase in response to RA treatment were observed in the PC-3 and SCC-25 cell lines. In contrast, the expression of the IL-1β gene was largely abolished in the prostate carcinoma LN-30 cell line.


A
Control
RA 2 hr
RA 4 hr
Cycl 2 hr
RA + Cycl 2 hr
Cycl 4 hr
RA + Cycl 4 hr
IL-1β
GAPDH

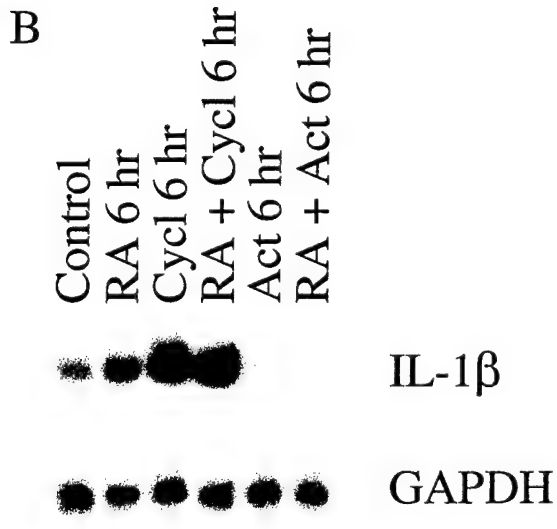

B
Control
RA 6 hr
Cycl 6 hr
RA + Cycl 6 hr
Act 6 hr
RA + Act 6 hr
IL-1β
GAPDH

Fig1

C

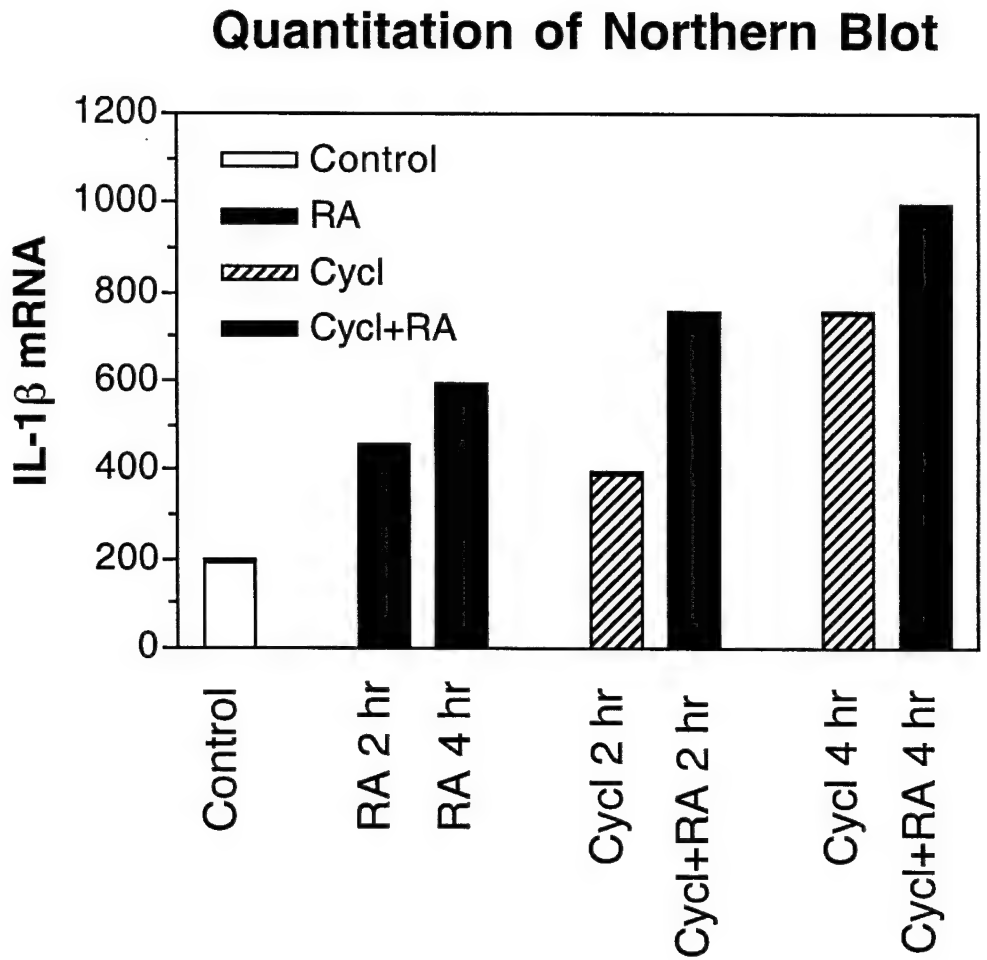

Quantitation of Northern Blot
IL-1β mRNA
1200
1000
800
600
400
200
0
Control
RA
Cycl
Cycl+RA
Control
RA 2 hr
RA 4 hr
Cycl 2 hr
Cycl+RA 2 hr
Cycl 4 hr
Cycl+RA 4 hr

Fig 1

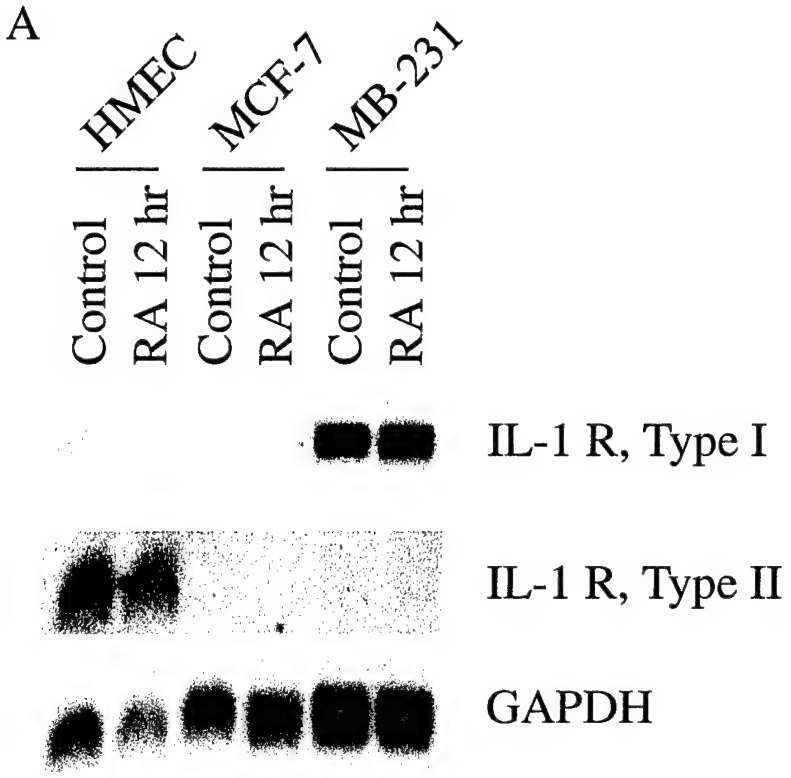

A
HMEC
MCF-7
MB-231
Control
RA 12 hr
Control
RA 12 hr
Control
RA 12 hr
IL-1 R, Type I
IL-1 R, Type II
GAPDH

Fig2

B

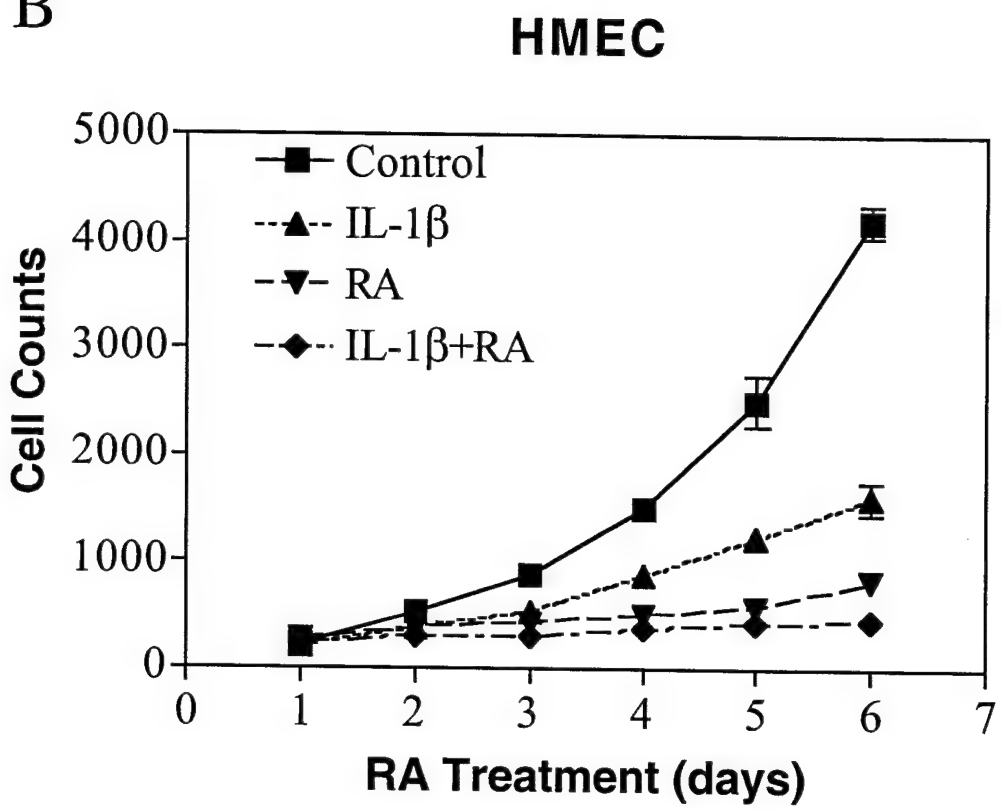

HMEC
Control
IL-1β
RA
IL-1β+RA
Cell Counts
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
RA Treatment (days)

Fig2

C

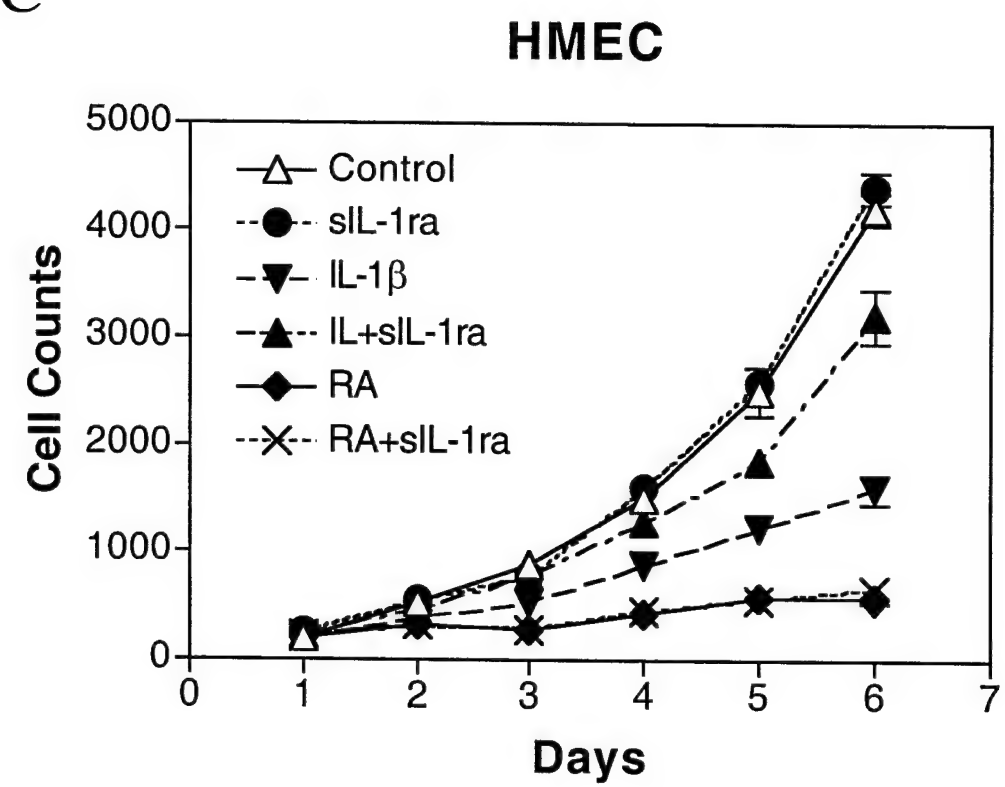
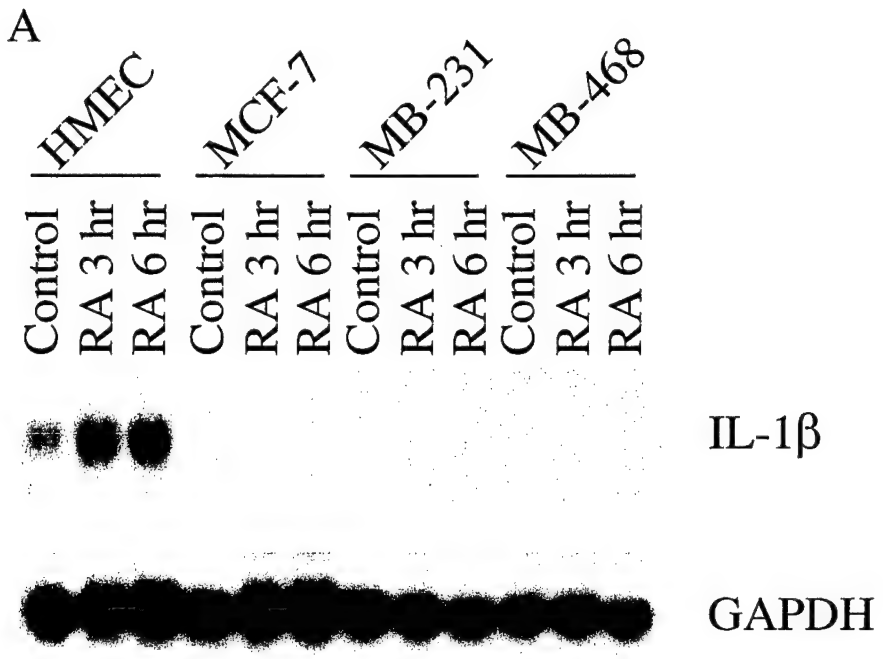

HMEC
Cell Counts
5000
4000
3000
2000
1000
0
Control
sIL-1ra
IL-1β
IL+sIL-1ra
RA
RA+sIL-1ra
0
1
2
3
4
5
6
7
Days

Fig 2


A
HMEC
MCF-7
MB-231
MB-468
Control
RA 3 hr
RA 6 hr
Control
RA 3 hr
RA 6 hr
Control
RA 3 hr
RA 6 hr
Control
RA 3 hr
RA 6 hr
IL-1β
GAPDH

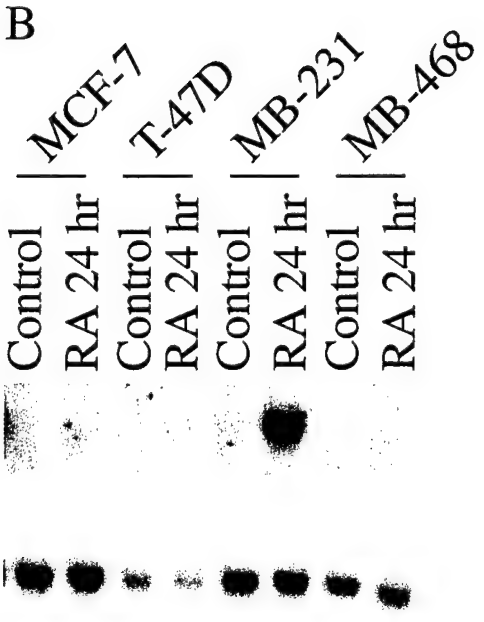

B
MCF-7
T-47D
MB-231
MB-468
Control
RA 24 hr
Control
RA 24 hr
Control
RA 24 hr
Control
RA 24 hr

Fig 3

A

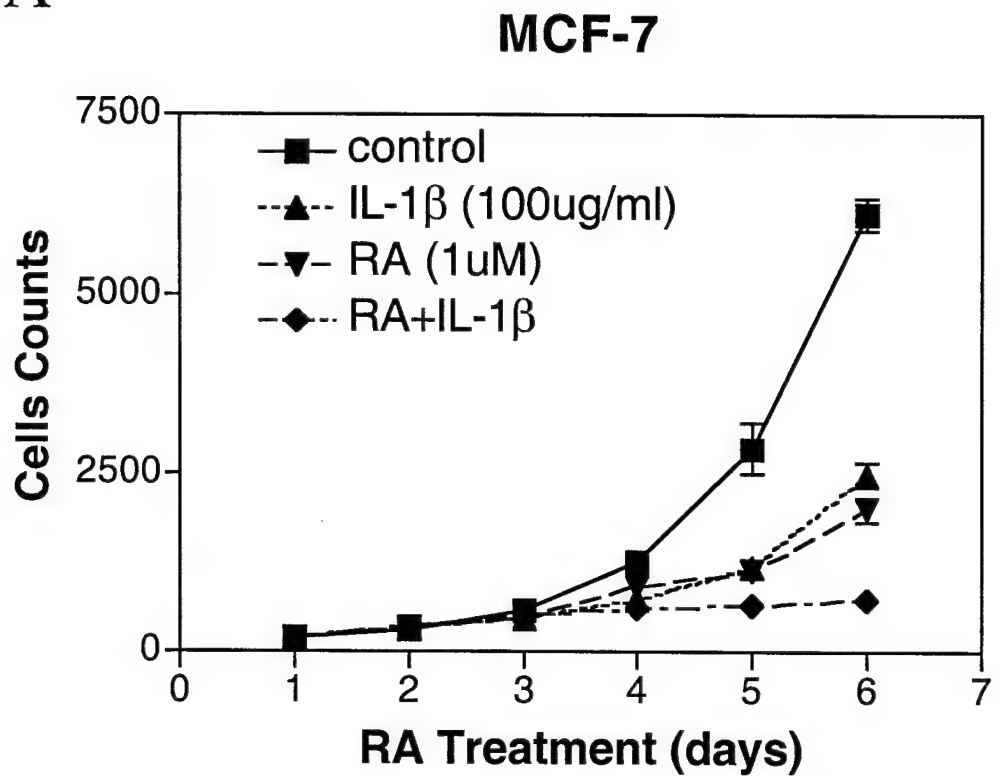

MCF-7
7500
5000
2500
0
Cells Counts
control
IL-1β (100ug/ml)
RA (1uM)
RA+IL-1β
0
1
2
3
4
5
6
7
RA Treatment (days)

Fig 4

B

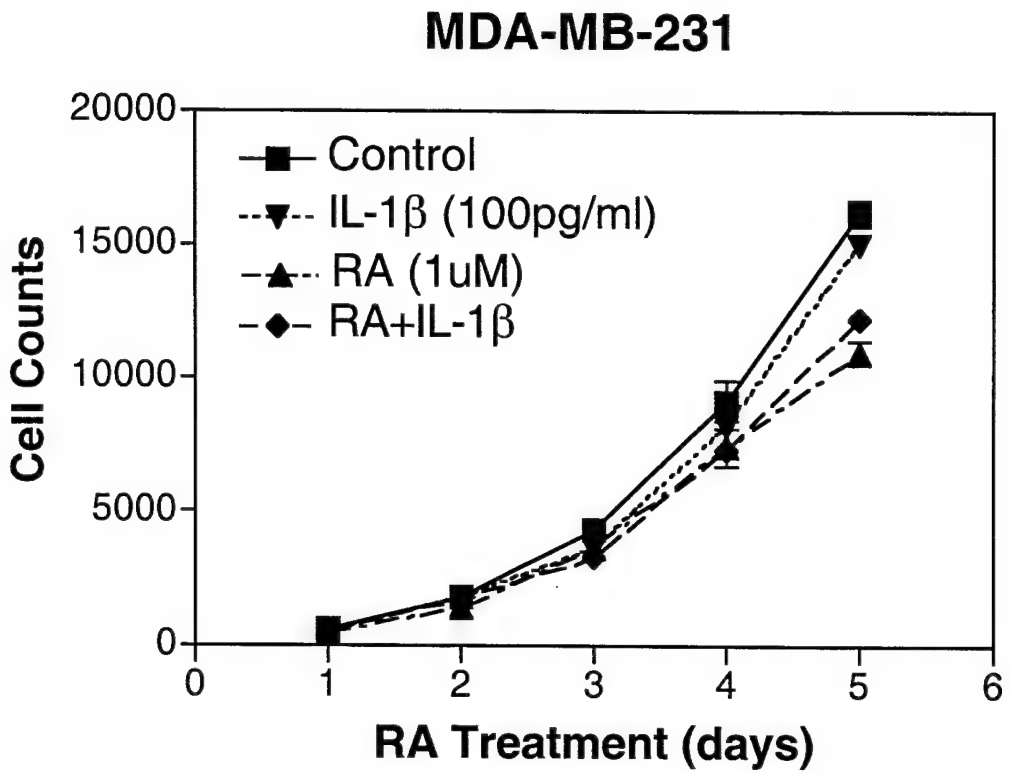

MDA-MB-231
20000
15000
10000
5000
0
Cell Counts
Control
IL-1β (100pg/ml)
RA (1uM)
RA+IL-1β
0
1
2
3
4
5
6
RA Treatment (days)

Fig 4

A

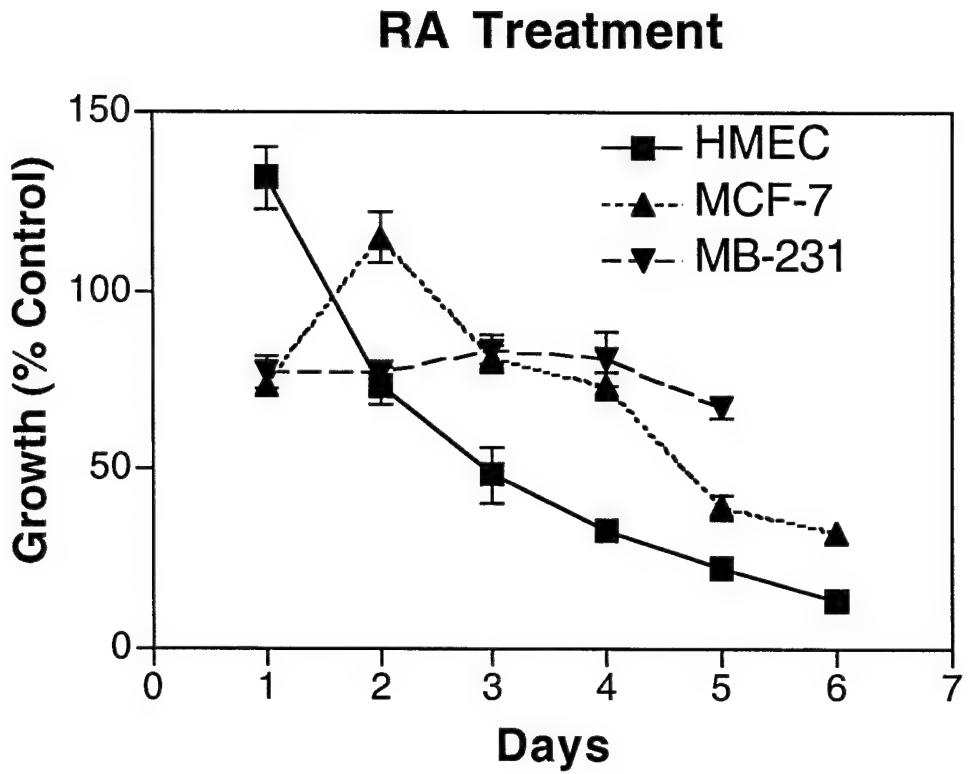

RA Treatment
Growth (% Control)
150
100
50
0
0
1
2
3
4
5
6
7
Days
HMEC
MCF-7
MB-231

Fig 5

B

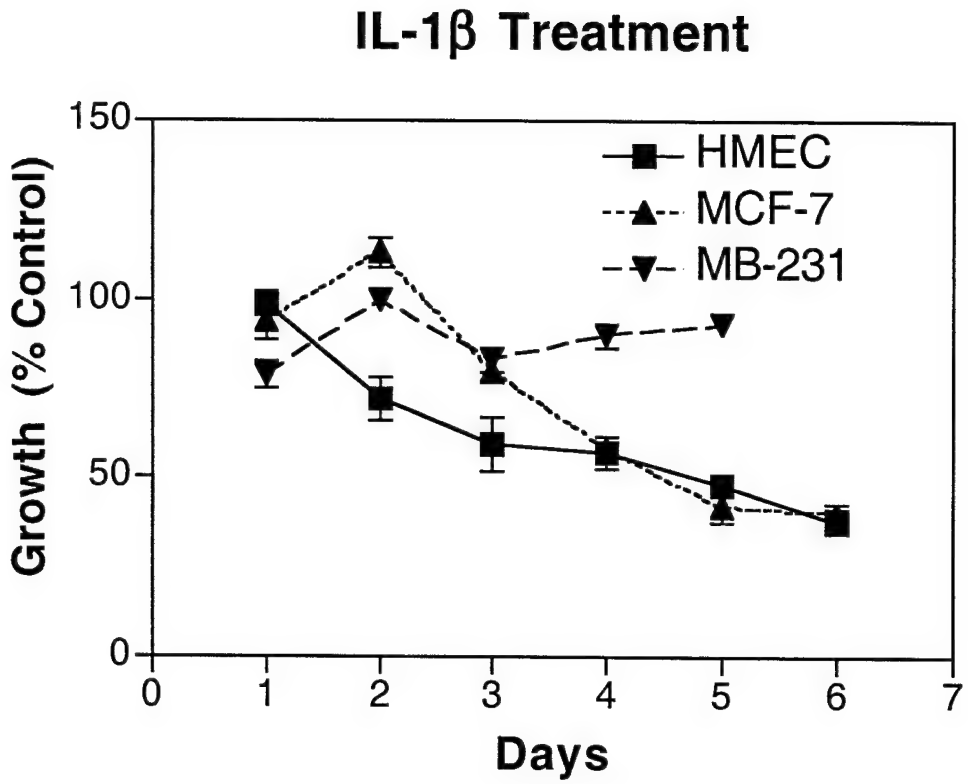

IL-1β Treatment
Growth (% Control)
150
100
50
0
0
1
2
3
4
5
6
7
Days
HMEC
MCF-7
MB-231

Fig 5

C

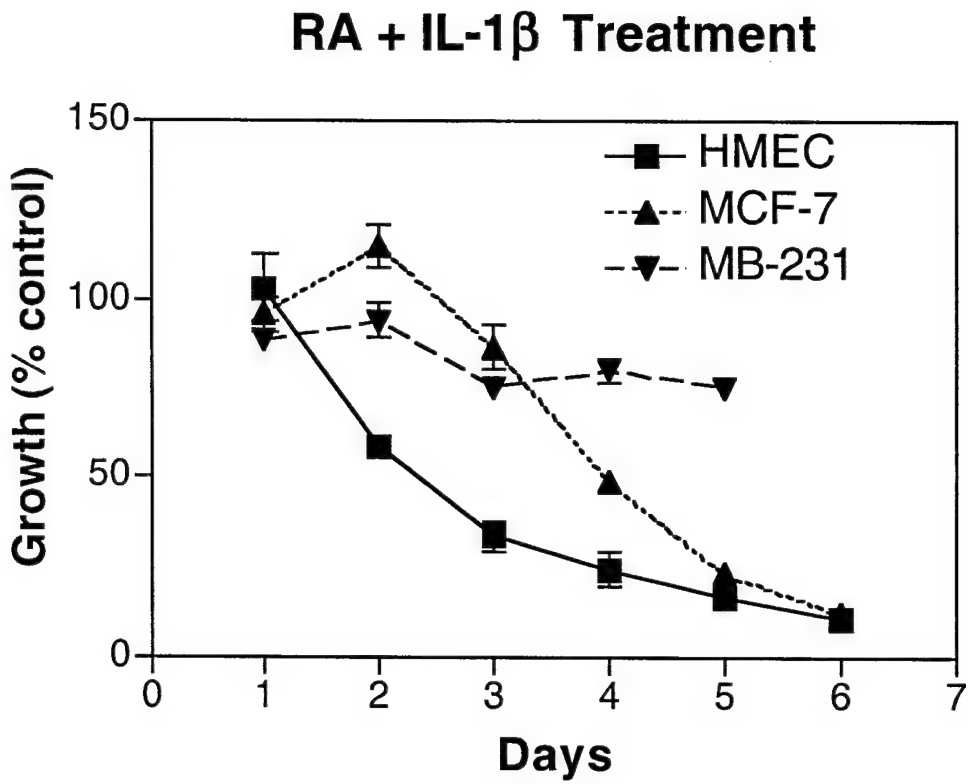

RA + IL-1β Treatment
Growth (% control)
150
100
50
0
0
1
2
3
4
5
6
7
Days
HMEC
MCF-7
MB-231

Fig 5

A

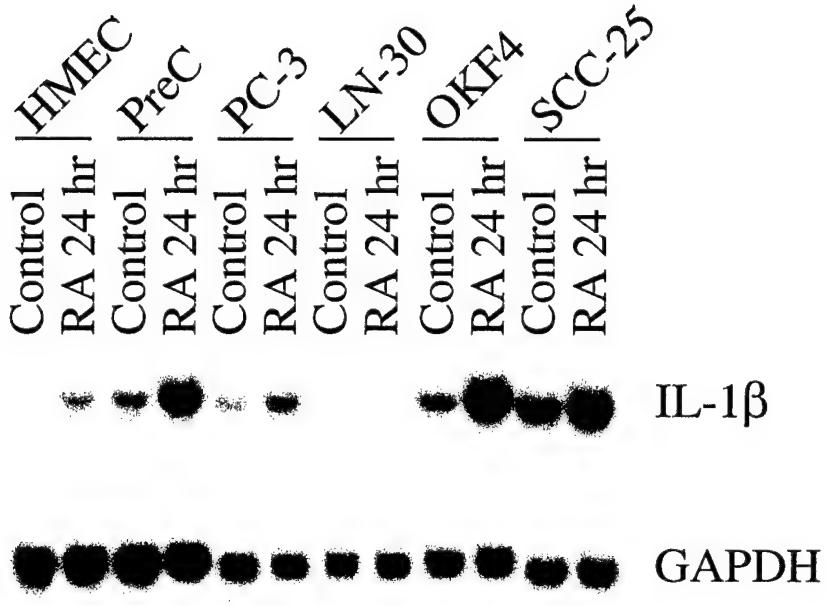


Fig 6

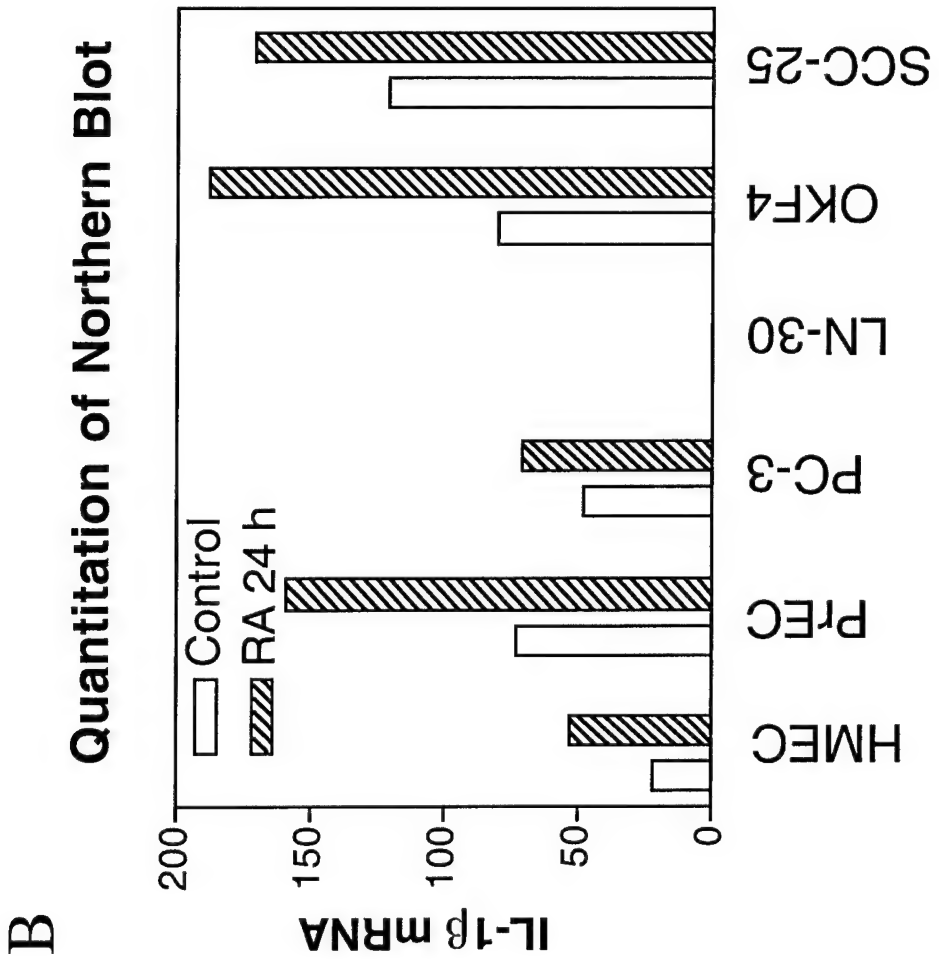

B
Quantitation of Northern Blot
IL-1β mRNA
200
150
100
50
0
Control
RA 24 h
HMEC
PrEC
PC-3
LN-30
OKF4
SCC-25

Fig 6

FIGURE LEGENDS

Figure 1. RA induces an increase in IL-1β mRNA levels. HMEC were grown in HEGM until they were ~80% confluent. Fresh medium was added to the HMECs 24 hours before the experiment. For the experiment, the cells were treated with RA (1 μM), cycloheximide (1 μM), and actinomycin D (2 μg/ml), as indicated. Total RNA was extracted from each plate of cells. RNA samples (20 μg total RNA) were loaded on a 1% agarose gel for Northern blot analysis. The human IL-1β cDNA fragment and GAPDH were labeled as probes. GAPDH was used as a loading control. This experiment was repeated three times and very similar results were obtained. One experiment is shown here. **A, B.** Northern blots; **C.** Quantitation of a single Northern blot; y axis, arbitrary units.

Figure 2. Inhibition of HMEC growth by RA and IL-1β. The HMECs were seeded in 24 well plates at $1x10^4$/well. Cells were grown in HEGM or HEGM supplemented with RA (1 μM), IL-1β (100 pg/ml), +/- GST-IL-1ra (IL1ra) (200 ng/ml) as indicated. The medium was changed every other day during the experiment. Cell numbers were counted each day and represented as the mean ± SD (n=3). **A.** Northern blots were hybridized to IL-1R type I and IL-R type II. Poly A^+ mRNAs (1 μg) isolated from HMEC, MCF-7 and MB-231 with or without RA treatment were loaded on a 1% agarose gel for Northern blot analysis. Human IL-1R type I (for all cell lines) and IL-1R type II cDNA fragments (for HMEC only) were labeled as probes. The probe was added at $2x10^6$ cpm/ml. Film was developed after 1 week exposure. This experiment was performed three times with similar results. One experiment is shown. **B.** Growth assays ±RA ± IL-1β. This experiment was performed three times and similar results were obtained. Standard error is shown (Y axis x100). **C.** The growth assays on the days 0-6 ± IL-1β ± RA ± sIL-1ra.. Standard error is shown (Y axis, x100).

Figure 3. IL-1β gene expression HMEC vs human breast carcinoma cell lines. All cells were grown in 100 mm plates until they were ~80% confluent. Fresh medium was added at 24-hour before the experiment. Total RNA was extracted from each cell line for Northern blot analysis. This experiment was performed four times, including one experiment with consensus medium. Similar results were obtained, and one result was shown. **A**. Northern blot, one day exposure. **B**. Northern blot, 14 days exposure.

Figure 4. Growth curves of MCF-7 and MDA-MB-231 cells with RA and/or IL-1β treatment. The growth of MCF-7 cells (**A**) and MDA-MB-231 cells (**B**) was examined in the presence of RA (1 μM) and/or IL-1β (100 pg/ml) as indicated. This experiment was performed three times and similar results were obtained. Standard errors are shown. Y axis, x100.

Figure 5. Comparison of growth inhibition of HMEC, MCF-7 and MB-231 cells with RA/or IL-1β treatment. **A.** Growth inhibition with RA treatment. **B.** Growth inhibition with IL-1β treatment. **C.** Growth inhibition with RA plus IL-1β treatment. Standard errors are shown.

Figure 6. RA-induced IL-1β gene expression in normal and malignant cell lines derived from epithelial tissues of prostate and oral cavity. All cells were grown in 100 mm plates until they were ~80% confluent. Fresh medium was added at 24 hours before the RA treatment. **A.** Northern blot analysis. **B.** Quantitative analysis of Northern blot. Y axis, arbitrary units. The band intensity was normalized by GAPDH within each cell line. This experiment was performed three times with similar results. One experiment is shown.

KEY RESEARCH ACCOMPLISHMENTS

1. IL-1β gene is a direct, downstream target gene of RA. RA activates the expression of IL-1β gene at transcriptional level.
2. The proliferation of HMEC is inhibited in the presence of RA and IL-1β. IL-1β inhibits the cell proliferation through a pathway that is independent to the RA pathway.
3. The RA-induced expression of the IL-1β gene is detected in ER negative MDA-MB-231 breast carcinoma cell line, but it is abolished in ER positive MCF-7 cell line.
4. The proliferation of MCF-7 cells is inhibited in the presence of RA and/or IL-1b. The proliferation of MDA-MB-231 line does not show significant response to RA and/or IL-1β treatment.

REPORTABLE OUTCOMES

1. Limin Liu and Lorraine Gudas. Overexpression of IL-1beta Gene Contributes to Retinoic Acid-induced Cell Growth Inhibition of Human Mammary Epithelial Cells. Abstract presentation at the 93rd Annual Meeting of American Association for Cancer Research, 2002, San Francisco, CA.
2. Limin Liu and Lorraine Gudas. Retinoic Acid Induces Expression of the Interleukin-1β Gene in Cultured Normal Human Mammary Epithelial Cells and Breast Carcinoma Lines. Manuscript was submitted for publication.

CONCLUSIONS

The present study indicates that all-*trans* retinoic acid *rapidly* increases the level of IL-1β mRNA in human mammary epithelial cells. Our results show that the RA-induction of IL-1β gene expression is completely inhibited by the transcription inhibitor actinomycin D, suggesting that RA regulates IL-1β gene expression by increasing the rate of transcription (Figure 1B). Our data also show that the RA induction of IL-1β gene expression is not blocked by the protein synthesis inhibitor cycloheximide (Figure1A), indicating that the regulation of IL-1β gene expression by RA does not require concurrent protein synthesis and that RA, via binding to its nuclear receptors, directly regulates IL-1β gene transcription. Sequence comparisons within the IL-1β promoter show that there are several potential RAREs within a 5 kb upstream sequence of the IL-1β gene (Liu, unpublished; e.g., the sequence AGGTCAAATGGTTCA was found between -4345 bp and -4331 bp upstream of the human IL-1β gene start site of transcription), but at present we do not know whether or not any of these putative RAREs are involved in the response to RA. We also observed that cycloheximide alone enhanced IL-1β gene expression in HMEC (Figure 1A and B). Although cycloheximide up-regulates the expression of some genes by stabilizing gene transcripts, cycloheximide may increase IL-1β gene expression by suppressing the synthesis of basal transcriptional repressors (Watkins, Hansen et al. 1999).

IL-1β regulates cell growth, differentiation, and apoptosis. Data from this present study and from others (Tsai and Gaffney 1987; Paciotti and Tamarkin 1991; Danforth and Sgagias 1993; Costantino, Vinci et al. 1996) indicate that IL-1β inhibits the proliferation of the ER positive MCF-7 cells. The inhibitory effect of IL-1β might be achieved via several pathways. IL-1β can block the insulin and insulin-like growth factor pathways by inhibiting the receptor kinase activity in MCF-7 cells and thereby can cause their growth arrest (Costantino, Vinci et al. 1996). In addition, IL-1β blocks estradiol-stimulated MCF-7 cell growth, which may result from the down-regulation of estrogen receptors (Danforth and Sgagias 1991) or from increased formation of estrogen sulfate (Purohit, de Giovani et al. 1999). Via binding to its receptors, IL-1β can activate MAP kinases and the NF-κB pathway. It was reported that p38 mitogen-activated protein (MAP) kinase was activated in response to RA treatment in the acute pro-myelocytic leukemia line NB-4, and in the breast carcinoma line, MCF-7 (Alsayed, Uddin et al. 2001), which demonstrates that RA can also activate MAP kinase pathways.

Our data show that the HMEC cells express IL-1 receptors (Figure 2A) and that exogenous IL-1β can efficiently suppress the growth of HMEC (Figure 2B), which raises a question as to whether the increased expression of IL-1β contributes to the RA-induced HMEC growth inhibition. However, we were not be able to block the RA-associated cell growth arrest using exogenous, recombinant soluble IL-1 receptor antagonist, while the HMEC growth arrest induced by exogenous recombinant IL-1β protein was markedly blocked by the sIL-ra fusion protein under the same conditions (Figure 2C). Therefore, our current data suggest that RA inhibits the growth of HMECs by a mechanism not involving the IL-1β signal transduction pathway. The biological role of the RA-induced IL-1β gene expression in these cells is unclear.

IL-1β may modulate the RA signal transduction pathway, and thus may indirectly regulate cell growth. Such signaling cross-talk is supported by other recent studies. The expression of the cellular retinoic acid binding protein type II gene in human keratinocytes was found to be regulated by IL-1α (Eller, Yaar et al. 1995); stimulation of vascular smooth muscle cells with IL-1β induced a substantial increase in retinol dehydrogenase-5 mRNA and a concomitant increase in the production of ligands for retinoic acid receptors (Gidlof, Romert et al. 2001); IL-1β enhanced the RA-induced expression of RARβ and RXRβ mRNAs as well as RA-mediated RXR response element binding (Nikawa, Ikemoto et al. 2001); and RXRα was shown to be a substrate for both mitogen-activated protein kinase kinase-4 (MKK4/SEK1) and c-Jun N-terminal kinase (JNK) (Lee, Suh et al. 2000).

Although the expression of the IL-1β gene in paclitaxel-treated (24 hr) MCF-7 cells was detected by RT-PCR in a previous study (White, Martin et al. 1998), our Northern blot analyses showed that the three breast carcinoma cell lines we examined lacked basal expression of the IL-1β gene (Figure 3 A, B). We did not detect expression of the IL-1β gene in the ER positive MCF-7 cells either with or without RA treatment. Since RA inhibited the proliferation of MCF-7 cells without inducing the expression of the

IL-1β gene, as was shown in the HMECs, these data also support the thesis that RA controls the growth of the MCF-7 cells through a mechanism which does not involve the IL-1β pathway.

Our results showed that RA induced IL-1β gene expression in two additional normal epithelial cell lines, the PrEC and the OKF4 cells, suggesting that the induction of IL-1β gene expression by RA could be a common event in epithelial cells (Figure 6). A dissociation between the synthesis of IL-1β mRNA and protein was found in certain circumstances (Schindler, Clark et al. 1990). However, most studies have shown that RA treatment induces a greater secretion of the mature IL-1β protein (Trechsel, Evequoz et al. 1985; Matikainen, Tapiovaara et al. 1994; Ross 1996), suggesting that transcription and translation of IL-1β are concordant in RA-treated HMECs. In our present study, we detected both basal expression and RA induction of the IL-1β gene in androgen receptor (AR) negative PC-3 prostate carcinoma cells, as well as in a human oral squamous cell carcinoma (SCC-25) line, but expression in the AR positive LN-30 cells was not detected either before or after RA addition (Figure 6A). These data are consistent with a previous study which showed that the IL-1β gene was only expressed in AR negative carcinoma cells, and not in AR positive carcinoma cells (Abdul and Hoosein 2000).

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